Carbohydrate Polymers 102 (2014) 622–626

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Immobilization of pectin degrading enzyme from *Bacillus licheniformis* KIBGE IB-21 using agar-agar as a support

Haneef Ur Rehman, Afsheen Aman, Raheela Rahmat Zohra, Shah Ali Ul Qader*

The Karachi Institute of Biotechnology and Genetic Engineering (KIBGE), University of Karachi, Karachi 75270, Pakistan

ARTICLE INFO

Article history:
Received 16 October 2013
Received in revised form
24 November 2013
Accepted 30 November 2013
Available online 7 December 2013

Keywords:
Pectinase
Immobilization
Agar-agar
Bacillus licheniformis

ABSTRACT

Pectinase from *Bacillus licheniformis* KIBGE IB-21 was immobilized in agar-agar matrix using entrapment technique. Effect of different concentrations of agar-agar on pectinase immobilization was investigated and it was found that maximum immobilization was achieved at 3.0% agar-agar with 80% enzyme activity. After immobilization, the optimum temperature of enzyme increased from 45 to 50 °C and reaction time from 5 to 10 minutes as compared to free enzyme. Due to the limited diffusion of high molecular weight substrate, K_m of immobilized enzyme slightly increased from 1.017 to 1.055 mg ml^{-1}, while V_{max} decreased from 23,800 to 19,392 μM min^{-1} as compared to free enzyme. After 120 h entrapped pectinase retained their activity up to 82% and 71% at 30 °C and 40 °C, respectively. The entrapped pectinase showed activity until 10th cycle and maintain 69.21% activity even after third cycle.

1. Introduction

Pectinases are heterogeneous group of enzymes involve in the degradation of long and complex carbohydrate polymer called pectin, present in the primary cell wall and middle lamellae of higher plants (Kashyap, Chandra, Kaul, & Tewari, 2000; Ridley, O'Neill, & Mohnen, 2001). Pectinases have various industrial applications as an integral part of fruit juices industry for the extraction and clarification of fruits juices and wine (Alkorta, Gabrbisu, Llama, & Serre, 1998; Kashyap, Vohra, Chopra, & Tewari, 2001). Pectinases have been used in textile industries for retting and degumming of plant fibers (Brühlmann, Kim, Zimmerman, & Fiechter, 1994; Cao, Zheng, & Chen, 1992; Henriksson, Akin, Slomezynski, & Eriksson, 1999; Kashyap et al., 2001). However, free enzymes are relatively unstable, costly and difficult to recover after industrial process. Enzyme immobilization is a technique that not only enhances the stability of enzyme but also helps in the separation of enzyme from the product; thereby minimize the chances of product contamination. In addition to this, enzyme immobilization facilitates the easy recovery of enzyme and enables the reuses of costly enzyme for continuous process.

Entrapment is one of the methods of immobilization that physically restrict the enzyme within confined space or network of polymer. Several supports have been used to immobilized enzymes such as nylon (Lozano, Manjio, Eomojaro, Canovas, & Iborra, 1987), ion-exchange resins (Kminkova & Kucera, 1983) and alginate (Qader & Aman, 2012; Tuoping, Suhong, Na, & Lirui, 2008). Agar is a polysaccharide composed of agarose, a linear polymer made up of the repeating monomeric unit of agarobiose. It has strong gelling ability, acid stable and shows no reactivity with protein (Prakash & Jaiswal, 2011). In the present study pectinase has been immobilized within agar-agar gel by entrapment method and kinetic behavior such as temperature, pH, K_m and V_{max} of immobilized pectinase was determined with reference to free enzyme. Finally the reusability and thermal stability of immobilized pectinase was investigated for industrial applications.

2. Material and methods

2.1. Pectinase production

Bacillus licheniformis KIBGE-IB21 strain isolated from rotten vegetable was used for pectinase production (Rehman, Qader, & Aman, 2012). The strain was inoculated in liquid medium containing citrus pectin (1.0%) as a sole carbon source and incubated at 37 °C for 48 h. After 48 h cells were separated by centrifugation at 15,000 rpm for 15 min at 4 °C.

2.2. Partial purification of pectinase

The cell free filtrate was partially purified using 50% ammonium sulphate saturation and kept for 12 h at 8–12 °C. After 12 h,

* Corresponding author at: The Karachi Institute of Biotechnology and Genetic Engineering (KIBGE), University of Karachi, Karachi-75270, Pakistan.
Tel.: +92 3212160109; fax: +92 21 32229310.
E-mail address: ali_kibge@yahoo.com (S.A.U. Qader).

0144-8617/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.11.073

precipitates were obtained by centrifugation (15,000 rpm) for 15 min at 4 °C. The precipitates were dissolved in glycine–NaOH buffer and dialyzed against the same buffer for overnight. The dialyzed enzyme solution was used for further study.

2.3. Effect of agar-agar concentration on pectinase immobilization

Agar-agar solution of different concentrations ranging from 2.0 to 5.0% were prepared in deionized water by heating and equal volume of enzyme solution (1:1 ratio) was mixed and immediately casted in preassembled glass plate. After solidification at the room temperature the gel was cut into small pieces and washed several times with deionized water and buffer (glycine–NaOH buffer 50 mM, pH 10.0) before use. These pieces were stored in glycine–NaOH buffer (50 mM, pH 10.0) at 4 °C for further studies.

2.4. Enzyme assay

The enzyme activity of free and immobilized pectinase were determined by 3′,5′-dinitrosalicylic acid method, using citrus pectin as a substrate and galacturonic acid monohydrate as standard (Miller, 1959). One unit of pectinase is defined as the amount of enzyme producing 1 μmole of galacturonic acid per minute under standard assay conditions.

2.5. Scanning electron microscopy

Scanning electron microscopy was performed to study the surface morphology of agar-agar gel before and after entrapment of pectinase. The dried samples were sputter-coated with gold and micrographs were taken using scanning electron microscope (JSM 6380A Jeol, Japan).

2.6. Effect of reaction time on immobilized pectinase

The effect of reaction time on immobilized pectinase activity was investigated by performing the immobilized enzyme–substrate reaction for different time intervals (5–60 min).

2.7. Effect of different pH on immobilized pectinase

The effect of pH on immobilized pectinase the activity was analyzed by measuring the reaction at different pH levels (5–12).

2.8. Effect of temperature on immobilized pectinase

The effect of incubation temperature on immobilized pectinase activity was determined by performing the reaction at different temperature ranging from 30 °C to 60 °C.

2.9. Kinetic studies of immobilized pectinase

The K_m and V_{max} values of immobilized pectinase was determined using Lineweaver–Burk plot by measuring reaction rates at different concentration of substrate ranging from 1.0 to 20 mg ml^{-1}.

2.10. Thermal stability of immobilized pectinase

The effect of temperature on stability of immobilized pectinase was analyzed by incubating immobilized enzyme in glycine–NaOH buffers (50 mM, pH 10.0) at different temperatures ranging from 20 to 60 °C for different time intervals. After every 24 h pectinase activity was estimated under standard assay conditions.

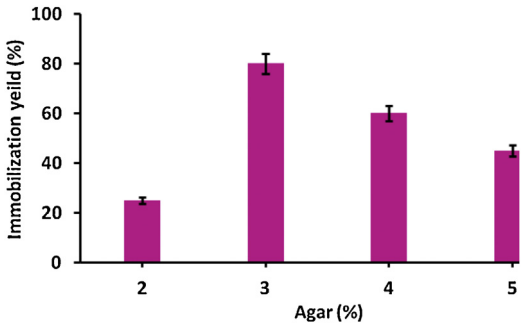

Fig. 1. Influence of agar-agar concentration on the immobilization of pectinase. Symbols (means ± S.E., $n = 6$) having similar letters are not significantly different from each other (Bonferroni test, $P < 0.05$).

2.11. Recycling of immobilized pectinase

The reusability of immobilized pectinase was determined in 10 cycles under optimized assay conditions. After each cycle the agar-agar immobilized enzyme were washed with glycine–NaOH buffer (pH 10) and deionized water to remove any residual substrate within/on the immobilized enzyme. The activity of immobilized enzyme in first run was defined as 100%.

3. Results and discussion

3.1. Effect of agar-agar concentration on immobilization of pectinase

The porosity of agar-agar matrix is responsible for the entrapment of enzyme and this porosity is directly related to concentration of agar-agar used. It has been observed that as the concentration of agar-agar was increased the percent relative activity of immobilized pectinase was also increased and maximum percent relative activity was obtained at 3.0% agar-agar (Fig. 1). Further increased in agar-agar concentration beyond 3.0%, it was found that the catalytic activity of immobilized pectinase was decreased and at 5% agar-agar more than 60% relative activity was decreased. When low concentration of agar-agar was used during immobilization very fragile and soft gel with larger pore size was formed which was not capable to entrap the enzyme and leakage of enzyme took placed from matrix during washing process. Similarly in case of using higher concentration of agar-agar the pore size of matrix was decreased and not allowed higher molecular weight substrate to diffuse easily into the matrix for reaction with entrapped enzyme. The maximum immobilization of enzyme not only depends on the concentration of agar-agar but also on the molecular size of enzyme and it was reported earlier that 4.0% of agar-agar was found suitable for the maximum immobilization of amylase (Prakash & Jaiswal, 2011). It was observed that agar-agar matrix gave higher immobilization efficiency as compared to the calcium alginate beads for the immobilization of same enzyme (Rehman et al., 2013).

3.2. Scanning electron microscopy

The scanning electron microscopy (SEM) was used to study the surface morphology of agar-agar matrix with and without entrapped pectinase. It was observed that the surface morphology of agar-agar matrix was changed after pectinase immobilization and these changes can easily be distinguished from scanning electron micrographs (Fig. 2). The micrographs clearly showed the pores on the surface of agar-agar matrix before immobilization of

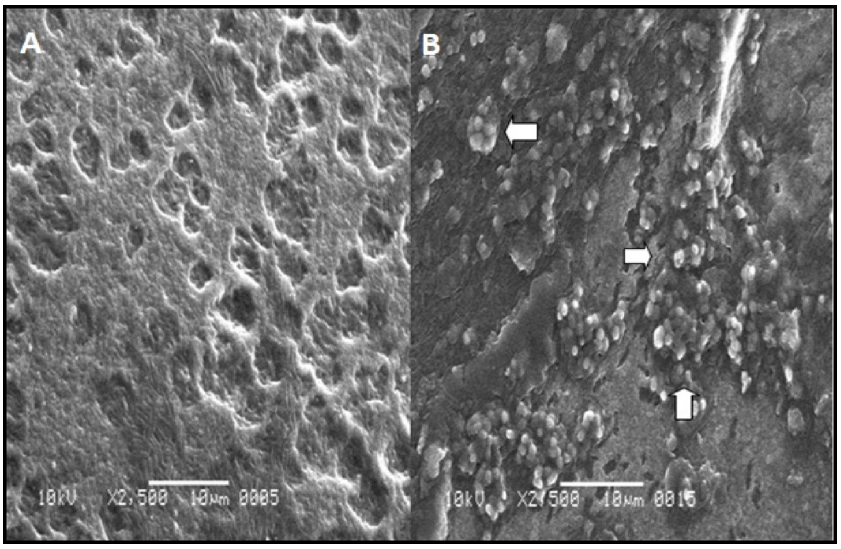

Fig. 2. Scanning electron micrographs of agar-agar matrix without (A) and with immobilized pectinase (B).

enzyme but after immobilization pores were covered with enzyme particles.

3.3. Effect of pH and temperature on immobilized pectinase

The effect of different pH on the catalytic activity of agar-agar entrapped pectinase was investigated by performing the enzyme assay in the reaction mixture having different pH ranging from 5.0 to 12.0 (Fig. 3A). It was observed that the agar-agar immobilized pectinase showed maximum activity at pH 9.0 as compared to free enzyme that showed maximum activity at pH 10. The alteration of optimum pH value of enzyme depends on nature of enzyme as well as the structure and composition of matrix used for immobilization, which might be responsible for deviation of the optimum pH value for maximum enzyme activity (Andriani, Sunwoo, Ryu, Prasetya, & Park, 2012). As compared to agar-agar matrix, no change in pH optima of pectinase was reported when pectinase immobilized within calcium alginate beads (Rehman et al., 2013). This change in pH in agar-agar entrapped pectinase might be due to the structural and conformational stability of pectinase within microenvironment of agar-agar matrix. The immobilized enzyme was less affected by the acidity of reaction mixture and showed higher activity in broad pH range. It was reported that when α-amylase entrapped within agar-agar matrix, the optimum pH was shifted from 5.5 to 7.0 (Prakash & Jaiswal, 2011). It was also reported that the optimum pH of pectinase was remained same but broaden to some extent after covalently immobilized with agar-agar through multipoint attachment (Tuoping et al., 2008).

The influence of temperature on the activity of agar-agar immobilized pectinase was investigated by performing the enzyme assay at different incubation temperature (30–60 °C) with reference to free enzyme. It was found that the soluble enzyme showed maximum activity at 45 °C, while agar-agar entrapped pectinase showed maximum activity at 50 °C (Fig. 3B). This slight increase in temperature of immobilized pectinase might be due to fact that immobilized enzyme required greater amount of activation energy as compared to free that was readily available in aqueous environment of reaction. The immobilization also increased the optimum range of temperature to some extent and immobilized pectinase showed greater relative activity at different temperatures as compared to free enzyme (Phadtare et al., 2002). It was reported that the optimum temperature of pectinase was increased up to 10 °C as compared to free enzyme when immobilized within calcium alginate beads and on agar-agar through covalent bonding (Rehman et al., 2013; Tuoping et al., 2008).

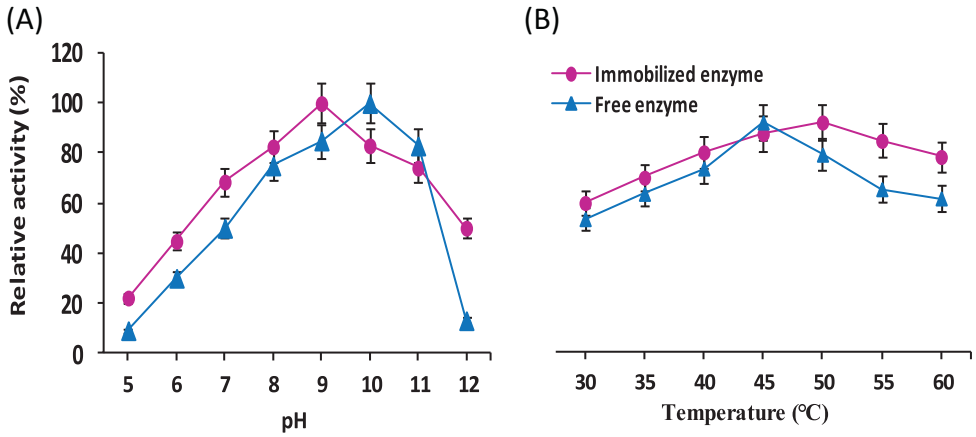

Fig. 3. Effect of pH on the activity of immobilized and free pectinase (A). Effect of different temperatures on the activity of agar-agar immobilized and free pectinase (B) (means ± S.E., $n = 6$).

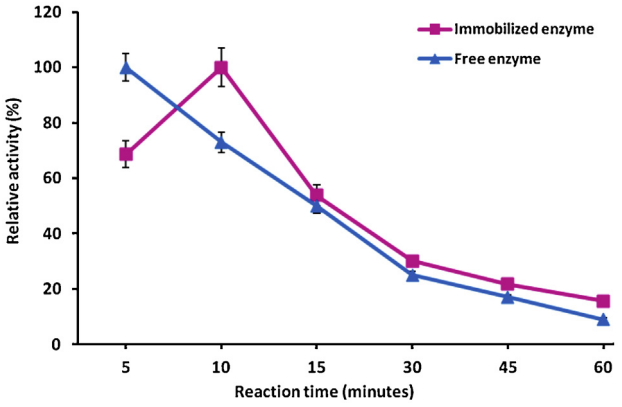

Fig. 4. Effect of reaction times on relative activity of immobilized and free enzyme at constant temperature (45 °C), pH (10.0) and substrate concentration (1.0%) (means ± S.E., $n = 6$).

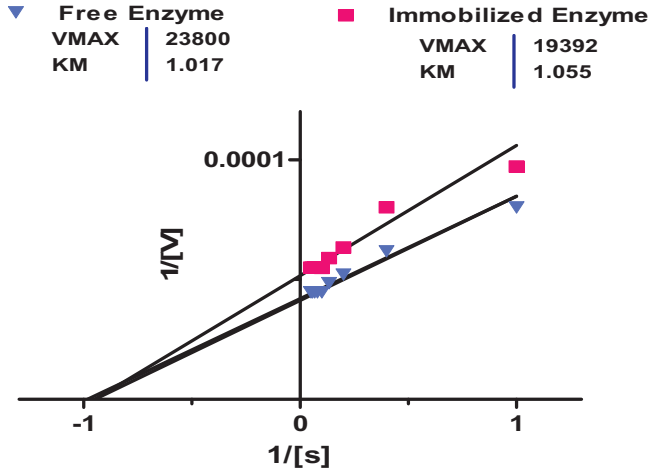

Fig. 5. Lineweaver–Burk plots of agar-agar matrix entrapped pectinase and free pectinase. Initial velocity data were fitted into Michaelis–Menten and Lineweaver–Burk equations using the Enzyme Kinetics module of GraphPad prism 5 software. Each data point was measured in triplicate.

3.4. Effect of reaction time on activity of immobilized pectinase

The effect of reaction time on activity of immobilized pectinase was studied by incubating of defined amount of immobilized pectinase and substrate for different time intervals using free enzyme as a control. It was observed that the immobilization increased the optimum reaction time from 5 to 10 min as compared with soluble enzyme (Fig. 4). This increase in reaction time might be due to the substrate diffusion limitation and substrate have to cross the polymeric network of matrix to react with the active site of enzyme as compared to free enzyme that readily available for a substrate to bind. Similar results were also observed in case of calcium alginate immobilization of pectinase and it was found that the reaction time was increased after immobilization from 5.0 to 10 min with reference to free enzyme (Rehman et al., 2013).

3.5. Kinetic parameters of immobilized pectinase

Kinetic parameters of agar-agar immobilized pectinase was investigated using different concentration (0.1–2.0%) of pectin as a substrate at constant temperature (50 °C) and pH (9) using Lineweaver–Burk plot with the reference to free enzyme. It was observed that the K_m value of pectinase was slightly increased and V_{max} values decreased after immobilization as compared to soluble enzyme (Fig. 5). The results indicated that due to the substrate diffusion resistance the agar-agar matrix lowered the accessibility of substrate to the active site of immobilized enzyme which ultimately reduced the affinity of enzyme as well as the velocity of enzymatic reaction. Similar findings was reported about the increased K_m and decreased V_{max} of enzymes after immobilization using different supports (Esawy, Gamal, Kamel, Ismail, & Abdel-Fattah, 2013; Lei & Bi, 2007; Sarıoğlu, Demir, Acar, & Mutlu, 2001).

3.6. Thermal stability of immobilized pectinase

The thermal stability of agar-agar immobilized pectinase was analyzed by pre-incubation of immobilized enzyme at various temperatures for different time intervals. The agar-agar immobilized pectinase showed higher thermal stability as compared free enzymes (Fig. 6A and B). The immobilized pectinase retained more than 80% residual activity at 30 °C and 70% residual activity at 40 °C after 120 h, while the free enzyme showed 40% to 30% residual activities at same conditions. Further increased in temperature up to 50 °C, the stability of free pectinase was decreased and free enzyme lost its complete activity after 72 h while immobilized pectinase still showed 50% residual activity at same temperature for same time. However, at 60 °C both the agar-agar immobilized as well as free enzyme lost their complete activity after 24 h. The increase of thermal stability of pectinase after immobilization within agar-agar matrix was might be due to the protection

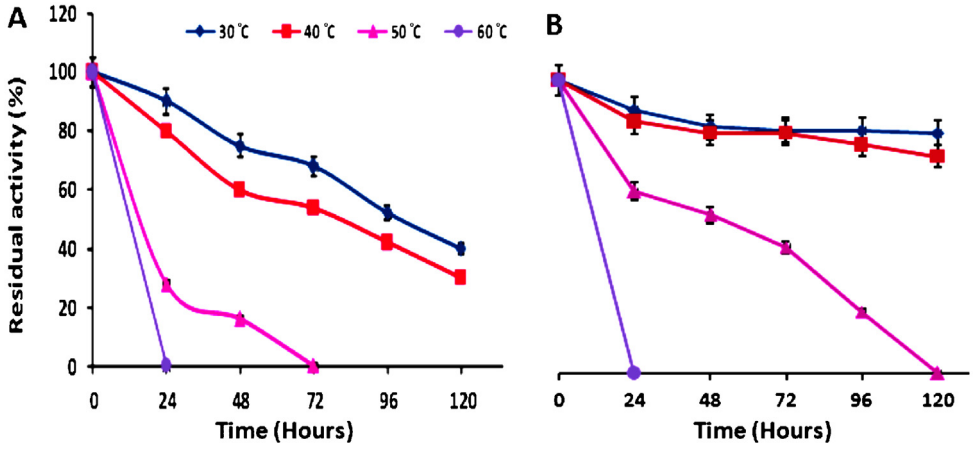

Fig. 6. Thermal stability of agar-agar immobilized pectinase (A) and free pectinase (B) at various temperatures for different time intervals. The first run for 0 h consider as 100% (means ± S.E., $n = 6$).

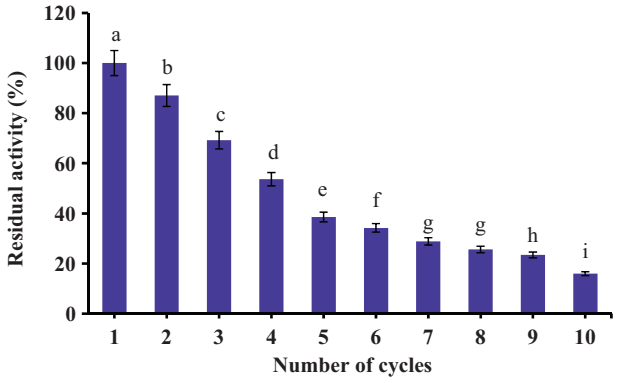

Fig. 7. Recycling efficiency of polyacrylamide gel immobilized pectinase in batch reactions (means ± S.E., $n = 6$). Symbols (means ± S.E., $n = 6$) having similar letters are not significantly different from each other (Bonferroni test, $P < 0.05$).

of immobilized enzyme from thermal denaturation effect of temperature by polymeric network of matrix (Tardioli, Zanin, & Moraes, 2006).

3.7. Reusability of immobilized pectinase

The recycling efficiency of agar-agar immobilized pectinase was monitored by reusing defined amount of immobilized pectinase and substrate and after completion of each batch, new substrate was added for new reaction until 10 batches of reactions (Fig. 7). It was observed that after first batch, the catalytic activity of agar-agar immobilized pectinase was started to decrease and approximately 15%, 25% and 40% activity was lost in second, third and fourth batch of reaction, respectively. After fifth batch slight decreased of activity was observed and it retained 20% of its initial activity even after repeating 10 times. This loss of enzyme activity might be due to leaching of enzyme from agar-agar matrix by excessive washing of pectinase immobilized agar-agar pieces after each batch or may be due to conformational changes of repeatedly reusing the enzymes. The results obtained are much higher as earlier reported in case of α-amylase entrapment in calcium alginate agar-agar (Riaz et al., 2009) and lower than that obtained using covalently bonding pectinase through multipoint attachment on activated agar-agar (Li et al., 2007). The activated agar-agar immobilized pectinase retained 81% residual activity after consecutively repeating the experiment 10 times (Li et al., 2007).

4. Conclusion

Pectinase have several applications in food industries including extraction and clarification of fruits and vegetable juices, extraction of vegetable oil, coffee and tea fermentation as well as clarification of wine. In current study, pectinase from *Bacillus licheniformis* KIBGE IB-21 was immobilized within agar-agar matrix using entrapment method. The 3.0% agar-agar was found to be optimum for maximum immobilization of enzyme. After immobilization enzyme–substrate reaction time increased from 5 to 10 min and temperature from 45 to 50 °C, whereas the pH was decreased from 10 to 9 as compared with free enzyme. The immobilized pectinase exhibited high reusability for pectin hydrolysis. The strategy of pectinase immobilization within agar-agar matrix permits good results in term immobilization and revealed promising applications in food industries.

References

Alkorta, I., Gabrbisu, C., Llama, J. M., & Serre, J. L. (1998). Industrial applications of pectic enzymes: A review. *Process Biochemistry*, *33*, 21–28.

Andriani, D., Sunwoo, C., Ryu, H. W., Prasetya, B., & Park, D. (2012). Immobilization of cellulase from newly isolated strain *Bacillus subtilis* TD6 using calcium alginate as a support material. *Bioprocess and Biosystems Engineering*, *35*, 29–33.

Brühlmann, F., Kim, K. S., Zimmerman, W., & Fiechter, A. (1994). Pectinolytic enzymes from *Actinomycetes* for the degumming of ramie bast fibers. *Applied and Environmental Microbiology*, *60*, 2107–2112.

Cao, J., Zheng, L., & Chen, S. (1992). Screening of pectinase producer from alkalophilic bacteria and study on its potential application in degumming of ramie. *Enzyme and Microbial Technology*, *14*, 1013–1016.

Esawy, M. A., Gamal, A. A., Kamel, Z., Ismail, A. S., & Abdel-Fattah, A. F. (2013). Evaluation of free and immobilized *Aspergillus niger* NRC1ami pectinase applicable in industrial processes. *Carbohydrate Polymers*, *92*(2), 1463–1469.

Henriksson, G., Akin, D. E., Slomezynski, D., & Eriksson, K. E. L. (1999). Production of highly efficient enzymes for flax retting by *Rhizomucor pusillus*. *Journal of Biotechnology*, *68*, 115–123.

Kashyap, D. R., Chandra, S., Kaul, A., & Tewari, R. (2000). Production, purification and characterization of pectinase from a *Bacillus* sp. DT7. *World Journal of Microbiology & Biotechnology*, *16*, 277–282.

Kashyap, D. R., Vohra, P., Chopra, S., & Tewari, R. (2001). Applications of pectinases in the commercial sector: A review. *Bioresource Technology*, *77*, 215–227.

Kminkova, M., & Kucera, J. (1983). Comparison of pectolytic enzymes covalently bound to synthetic ion exchangers using different methods of binding. *Enzyme and Microbial Technology*, *5*, 204–208.

Lei, Z., & Bi, S. (2007). The silica-coated chitosan particle from a layer-by-layer approach for pectinase immobilization. *Enzyme and Microbial Technology*, *40*, 1442–1447.

Li, T., Wang, N., Li, S., Zhao, Q., Guo, M., & Zhang, C. (2007). Optimization of covalent immobilization of pectinase on sodium alginate support. *Biotechnology Letters*, *29*, 1413–1416.

Lozano, P., Manjio, A., Eomojaro, F., Canovas, M., & Iborra, J. L. (1987). A cross-flow reactor with immobilized pectolytic enzymes for juice clarification. *Biotechnology Letters*, *9*, 875–880.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Analytical Chemistry*, *31*, 426–428.

Prakash, O., & Jaiswal, N. (2011). Immobilization of a thermostable α-amylase on agarose and agar matrices and its applications in starch stain removal. *World Applied Sciences Journal*, *13*(3), 572–577.

Phadtare, S., Parekh, P., Gole, A., Patil, M., Pundle, A., Prabhune, A., & Sastry, M. (2002). Penicillin G acylase-fatty lipid biocomposite films show excellent catalytic activity and long term stability/reusability. *Biotechnology Progress*, *18*, 483–488.

Qader, S. A. U., & Aman, A. (2012). Low molecular weight dextran: Immobilization of cells of *Leuconostoc mesenteroides* KIBGE HA1 on calcium alginate beads. *Carbohydrate Polymers*, *87*, 2589–2592.

Rehman, H. U., Aman, A., Silipo, A., Qader, S. A. U., Molinaro, A., & Ansari, A. (2013). Degradation of complex carbohydrate: Immobilization of pectinase from *Bacillus licheniformis* KIBGE-IB21 using calcium alginate as a support. *Food chemistry*, *139*, 1081–1086.

Rehman, H. U., Qader, S. A. U., & Aman, A. (2012). Polygalacturonase: Production of pectin depolymerising enzyme from *Bacillus licheniformis* KIBGE IB-21. *Carbohydrate polymers*, *90*, 387–391.

Riaz, A., Qader, S. A. U., Anwar, A., & Iqbal, S. (2009). Immobilization of a thermostable α-amylase on calcium alginate beads from *Bacillus subtilis* KIBGE-HAR. *Australian Journal of Basic and Applied Sciences*, *3*, 2883–2887.

Ridley, B. L., O'Neill, M. A., & Mohnen, D. (2001). Pectins: Structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry*, *57*, 929–967.

Sarıoğlu, K., Demir, N., Acar, J., & Mutlu, M. (2001). The use of commercial pectinase in the fruit juice industry, part 2: Determination of the kinetic behaviour of immobilized commercial Pectinase. *Journal of Food Engineering*, *47*, 271–274.

Tardioli, P. W., Zanin, G. M., & Moraes, F. F. D. (2006). Characterization of thermoanaerobacter cyclomaltodextrin glucanotransferase immobilized on glyoxyl-agarose. *Enzyme and Microbial Technology*, *39*(6), 1270–1278.

Tuoping, L., Suhong, L., Na, W., & Lirui, T. (2008). Immobilization and stabilization of pectinase by multipoint attachment onto an activated agar-gel support. *Food Chemistry*, *109*, 703–708.